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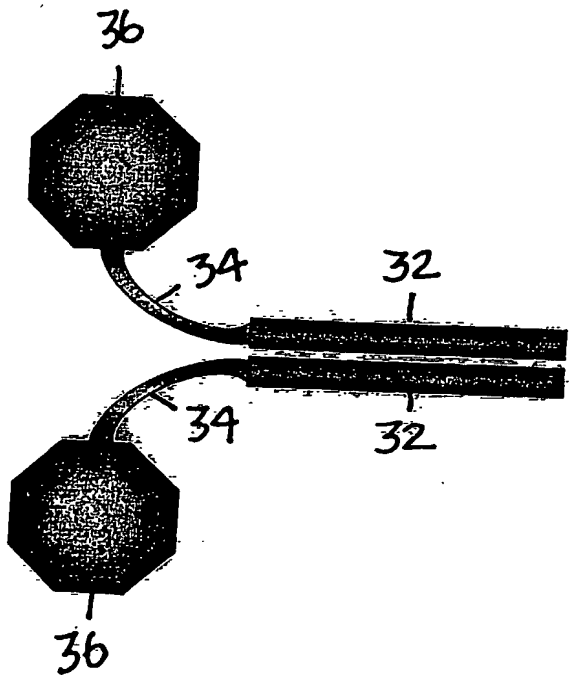
Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i)) for the following designation US*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations*

[Continued on next page]

(54) Title: COMPOSITION AND METHOD FOR ENHANCING IMMUNE RESPONSE



(57) Abstract: A composition and method for enhancing immune response in a living organism is disclosed. In particular, the present disclosure provides an adjuvant peptide for use in raising an immune response to an antigen. The adjuvant peptide is selected from a group of peptides with an HIV-related sequence. Additionally, the adjuvant peptide can comprise a fusion-protein that acts as a mucosal adjuvant. The adjuvant peptide can be transformed into one or more living cells, such that the mucosal adjuvant can be produced in living cells and then administered by systemic, mucosal or epidermal delivery.

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COMPOSITION AND METHOD FOR ENHANCING IMMUNE RESPONSE

CLAIM TO DOMESTIC PRIORITY

[0001] The present non-provisional patent application claims priority to provisional application serial no. 60/362,247, entitled "A Novel Oral Adjuvant: Immunostimulatory Effect of HIV-Derived P1 Peptide," filed on March 6, 2002, by Nobuyuki Matoba, Charles J. Arntzen and Tsafrir Mor.

FIELD OF THE INVENTION

[0003] The present invention relates generally to a composition and method for enhancing immune responses, and more specifically, to a composition and method using HIV-related peptides as an agent to increase immunogenic responses and for delivering fusion proteins to animal cells.

BACKGROUND OF THE INVENTION

[0004] Most currently available vaccines consist of killed or live-attenuated pathogens delivered by injection. Despite their success in preventing disease, compelling conceptual, technical and economical reasons exist to seek alternatives to traditional "Jennerian" vaccines.

[0005] Vaccines delivered parenterally require injections that must be given by medically trained personnel. Additionally, injection risks possible transmission of infection. Finally, parenteral delivery of vaccines invokes a systemic response, but not a mucosal response.

[0006] Subunit vaccines, especially those vaccines that target the mucosal immune system, are viable, safe and effective alternatives. Muscosal vaccines require do not require injection; thus, risk of transmission of infection is minimal. Finally, mucosal vaccines elicit immune response both systemically and mucosally.

[0007] Additionally, recent breakthroughs suggest that vaccines can be produced in edible tissues of transgenic plants that can then be orally immunogenic. The concept of using transgenic plants as vectors for the production and delivery of edible vaccines has been previously demonstrated.

[0008] However, to be effective, mucosal subunit vaccines often need to be co-administered with an "adjuvant." An "adjuvant" is an immunostimulatory agent that would enhance the specific immune responses against the vaccine candidate.

[0009] Therefore, a need exists for an immunostimulatory, mucosally-active composition that can be used as a systemic, mucosal, or epidermal adjuvant.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] FIG. 1 depicts the structure of a human immunodeficiency virus (HIV);

[0011] FIG. 2 depicts the structure of an adjuvant according to one embodiment;

[0012] FIG. 3 illustrates an ELISA determination of anti-CTB antibodies following immunization by gavage;

[0013] FIG. 4 shows end point titers of anti-CTB antibodies;

[0014] FIG. 5 illustrates reciprocal dilution of serum IgG_1.

[0015] FIG. 6 illustrates subclass titers of total serum IgG, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgA.

[0016] FIG. 7 is a flowchart illustrating immune response of Th1 and Th2.

[0017] FIG. 8 depicts the synthesis of a plant-expression optimized DNA molecule encoding for the P1 peptide;

[0018] FIG. 9 depicts maps of plasmids comprised of DNA sequences of CTB, P1, CTB-P1 fusion, and for the plant-expression of the CTB-P1 fusion;

[0019] FIG. 10 is a flowchart illustrating the construction of a CTB-P1 fusion protein for plant-expression;

[0020] FIG. 11 depicts maps of plasmids for expression of CTB-P1 fusion protein and CTB in tomato;

[0021] FIG. 12 shows the expression of CTB-P1 fusion protein in *E. Coli* cells; and

[0022] FIG. 13 illustrates an ELISA detection of anti-CTB and anti-P1 in *E. Coli* cells.

SUMMARY OF THE INVENTION

[0023] The present invention provides a composition and method for enhancing immune response in living organisms, for example, in humans. In one embodiment, and by way of example only, the composition includes, a peptide that when administered to a living organism, enhances the organism's immune response. The composition may also include an antigen, for example, a cholera toxin. The composition may further include the peptide and the antigen together as a fusion protein. The adjuvant peptide may function as a systemic, mucosal or epidermal adjuvant.

[0024] In another exemplary embodiment, the adjuvant peptide may be encoded by a genetically-modified living cell. The genetically-modified living cell may also encode an antigen. The peptide and antigen may also be encoded as a fusion protein.

[0025] Other independent features and advantages of the method for decreasing nicotine use in living organisms will become apparent from the following detailed description, taken in conjunction with the accompanying drawings which illustrate, by way of example, the principles of the invention.

DETAILED DESCRIPTION OF VARIOUS EMBODIMENTS

[0026] This description discloses a composition and method for enhancing immune response in living organisms by administering an oral, mucosal or epidermal adjuvant comprised of one or more HIV-related peptides.

[0027] FIG. 1 depicts the structure of an HIV retrovirus. HIV retrovirus 10 is an enveloped retrovirus. HIV retrovirus 10 is comprised of a viral membrane 12, ampiphilic regions 14, charged helices 16, calcium (Ca^{2+}) binding sites 18, gp41 subunits 20, and gp120 subunits 22. Adjuvant peptide 24 facilitates HIV transcytosis across muscosal barriers toward the serosal environment by binding to galactosyl ceramide (GalCer) on the surface of mucosal epithelial cells.

[0028] Adjuvant peptide 24 comprises 36 amino acids (SEQ. ID. NO: 1) correspondes to a portion of the gp41 envelope. This peptide includes a conserved epitope (SEQ. ID. NO: 2), which is recognized by the neutralizing human IgG 2F5 and secretory IgAs that functionally neutralize HIV transcytosis through epithelial cells. The conserved aromatic residues are important for GalCer binding.

[0029] FIG. 2 depicts the structure of an adjuvant 30 according to one embodiment. Adjuvant 30 comprises peptides 32, linkers 34, and cargo proteins 36. However, an alternate embodiments envisions adjuvant 30 comprising at least peptides 32, but not necessarily linkers 34 and cargo proteins 36. Peptides 32 may comprise adjuvant peptides as one or more portions of P1 peptides, P5 peptides, or

their functional equivalents. In one embodiment, cargo protein 36 is an antigen, for example cholera toxin. In an alternate embodiment, cargo protein 36 is any protein to be delivered to an animal cell.

[0030] According to one embodiment, an adjuvant peptide is a portion of the P1 peptide, HIV envelope protein gp41, which includes the conserved epitope, lectin binding site (SEQ. ID. NO: 2). According to an alternate embodiment, the adjuvnat peptide is a portion of the P5 peptide, HIV envelope protein gp41 which includes the P1 peptide and a calcium binding site (residue number 622-684). P1 and P5 peptides also include their functional equivalents.

[0031] Functional equivalents of adjuvant peptides include peptides or portions of larger proteins with overall sequence or structural similarity to P1 or P5 peptides, and their derivatives, which allow the functionality disclosed here, including, but not limited to, one or more of the following: the enhancing immune response, GalCer binding, binding to the surface of cells containing GalCer, endocytosis to such cells or transcytosis across a tight cell barrier.

[0032] Examples of functional equivalents include portions of variants of gp41 in naturally occurring strains of HIV or in laboratory-derived strains of HIV, including, but not limited to, site-directed mutated versions of the gp41 portion of the molecule. Specific, non-limiting, examples of functional equivalents are HIV-1 isolate MN clone V5 (SEQ. ID. NO: 4), HIV-1 isolate 593 clone (SEQ. ID. NO: 5), HIV-1 isolate 98BRRS012 (SEQ. ID. NO: 6), and HIV-1 isolate 19242v3.20 (SEQ. ID. NO: 7).

[0033] Adjuvant 30 is capable of mucosal administration. Mucosal administration includes oral, nasal, vaginal, or rectal administration. Adjuvant 30 is also capable of functioning as a systemic, mucosal, or epidermal adjuvant.

[0034] EXAMPLE 1

[0035] Example 1 demonstrates that adjvuant peptide enhances immune responses against cholera toxin B subunit by mucosal co-administration of adjuvant peptide and cholera toxin B subunit. Synthetic adjuvant peptide (SEQ.

ID. NO: 3) with a C-terminal $CONH_2$, was synthesized by Eurogentec (Belgium) and by the Protein Chemistry Laboratory at Arizona State University. A cystine residue was added to the beginning of SEQ. ID. NO: 1 to allow for dimerization (residue 649). Cholera Toxin B (CTB) subunit was chosen for co-administration because it is non-toxic and it is a strong mucosal adjuvant. Additionally, CTB binds to G_{M1} ganglioside whereby being able to target the fused antigen to mucosa.

[0036] Synthetic adjuvant peptide 30 micrograms (μg), adjuvant peptide plus Cholera-Toxin B subunit (CTB) (30 and 70 μg, repsectively), and CTB (70 μg) were given orally to CD1 female mice (6-7 weeks old) by a gastic feeding tube on day one, eight, and fifteen. The serum, fecal pellets and vaginal secretions were collected prior to and on the second, third and fourth weeks after the first administration. Levels of anti-adjuvant peptide and anti-CTB antibodies were determined by ELISA in each sample.

[0037] FIG. 3 illustrates an ELISA determination of anti-CTB antibodies following immunization by gavage of CTB (70μg), CTB+P1 (70μg and 30 μg, respectively) or P1 (30 μg). Mice were gavaged on days indicated by arrows and samples of serum (A), fecal (B), and vaginal (C) secretions were collected when indicated. Serum (A) detected systematic levels. Fecal (B) and vaginal (C) both detected mucosal levels.

[0038] Samples were serially diluted in phosphate buffered saline containing 0.05% Tween-20 (PBST) containing 1% nonfat dry milk. Plates were coated with CTB overnight at 4°C, blocked with PBST containing 5% nonfat dry milk and then incubated with samples. Antibodies were detected by horseradish peroxidase-conjugated secondary antiisotypic antisera against the appropriate mouse antibodies (rabbit anti-mouse total IgG from CalBiochem, and the following anti mouse antisera: Anti-IgG_1, anti-IgG_{2a}, anti-IgG_{2b}, anti-IgG_3 from Santa Cruz Biotechnology; and anti-IgA from Sigma. FIG. 3A-3C shown maximal dilutions that allowed quantification.

[0039] FIG. 4 illustrates end point of anti-CTB antibodies four weeks after immunization. Chemiluminescent ELISA was conducted as described in FIG. 3. Titers in FIG. 4 are defined as reciprocals of the highest dilution giving a positive A_{490} reading above 0.1. FIG. 5 illustrates, for example, reciprocal dilution of serum IgG_1. FIG. 6 illustrates subclass titers of total IgG, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgA.

[0040] While in FIG. 4 antibody titers were below detection levels, co-administration of P1 and CTB to mice resulted in significantly higher titers of anti-CTB antibodies as compared to mice that were given CTB alone. Specifically, in FIG. 3, the level of fecal and vaginal anti-CTB IgA in the second and third week and serum andti-CTB in the second, third and fourth week appeared to be higher in mice fed P1 with CTB than in mice fed only CTB. Moreover, as illustrated in FIG. 6, co-administration of P1 with CTB resulted in increasing all serum anti-CTB IgG subclass (IgG_1, IgG_{2a}, IgG_{2b}, $IgG_{3)}$ titers by five to ten times in the fourth week, as compared to administration of CTB alone, as shown in FIG. 4.

[0041] Therefore, P1 peptide was shown to augment the production of mucosal IgA and serum IgG to co-administered CTB. Because CTB is a strong mucosal immunogen by itself, the increase of both anti-CTB IgG_1 and IgG_{2a} levels suggest that the immune enhancement effect of P1 peptide is attributable to activating both Th1 and Th2 response. Th1 and Th2 response is illustrated in FIG. 7. IgG2a 40 effects T1 response 38 through cell-mediated immunity, targeting intracellular pathogens 42. Antibodies 46, such as IgG_1 and IgA, affect Th2 response 44 targeting extracellular parasites, viruses and bacteria 48. Secondly, P1 peptide did not induce antibody production against itself, even in the presence of CTB. Therefore, P1 peptide can be used a mucosal adjuvant to enhance immune response in living organisms.

[0042] EXAMPLE 2

[0043] In example 2, plasmids were created for the co-expression of adjuvant peptide and GFP in transgenic plants for oral delivery. FIG. 8 depicts the synthesis of a plant-expression optimized DNA molecule encoding for an adjuvant peptide-GFP fusion protein. The sequence coding for adjuvant peptide was inserted behind a DNA spacer encoding a Glycine-Proline-Glycine-Proline (GPGP) hinge. A *BsrGI-SacI* fragment of this plasmid was cloned in behind a 35 S Promoter. A *PstI-EcoRI* fragment contains the plant expression cassette. FIG. 8 represents a model delivery system for using fusion proteins to deliver cargo proteins to an animal cell.

[0044] FIG. 9 depicts maps of plasmids comprised of DNA sequences of CTB, P1, CTB-P1 fusion and for the plant-expression of the CTB-P1 fusion protein. A plant-expression optimized DNA molecule encoding for P1 peptide was synthesized. The sequence was inserted behind a portion of the gene encoding the C-terminus of the CTB molecule behind a DNA spacer encoding a Glycine-Proline-Glycine-Proline (GPGP) hinge. An endoplasmic reticulum (ER) retention signal was engineered at the Carboxyl-Terminus. The PCR product was closed into the cloning vector TOPO2.1 (Invitrogen) to create pTM058.

[0045] Still referring to FIG. 9, a *HindIII-SacI* fragment of this plasmid was then cloned into pTM042 to create a gene encoding a Carbosyl-terminus fusion of CTB and the P1 peptide in the plasmid pTM065. A *BspHI-SacI* fragment of this plasmid was cloned into pIBT210.1 (Haq, et al. 1995) behind a CaMV35S promoter and the 5' UTR of Tobacco Etch Virus and in front of the 3' UTR of the soy bean *vsp*B gene to form pTM066. A *PstI-EcoRI* fragment containing the plant expression cassette was cloned into the T_1 plasmid derivative pGPTV-Kan (Becker, et al. 1992) to form pTM067 (not shown).

[0046] FIG. 10 is a flowchart illustrating the steps involved in creating a CTB-P1 fusion protein. In step 50, CTB (from *HindIII* site to the 3' end)-P1 fusion gene is designed and synthesized, a length of 234 base pairs (bp) (SEQ. ID. NO: 8). Next, in step 52, the CTB-P1 fusion gene is cloned into TOPO.

[0047] In step 54, the sequence is corrected by PCR-based site-directed mutagenesis to form pTM58 (pTM058). In step 56, the synthetic gene is cut out by *HindIII* and *Sac I*. Then in step 58, the synthetic gene is cloned into *HindIII-SacI* site of pTM42 (pTM042). This represents the complete CTB-P1 fusion gene.

[0048] The CTB-P1 fusion gene is then cut out by *BspHI* and *SacI* in step 60. Finally, in step 62, the cut out CTB-P1 fusion gene is cloned into the *NcoI-SacI* site of pTM38. Thus, step 62 clones the CTB-P1 fusion gene into the plant expression cassette. The CTB-P1 fusion gene encodes the CTB-P1 fusion protein (SEQ. ID. NO: 9).

[0049] FIG. 11 illustrates an example for a construct for a potentiated edible vaccine. In step 80, pTM086 containing the CTB-P1 fusion gene and the plant expression cassette was cloned into the T_1 plasmid derivative pGPTV-Kan (Becker, et al. 1992). The plasmid is then transformed into Agrobacterium (LBA4404) in step 82. Finally, in step 84, the Agrobacterium is transformed into a tomato, for example MicroTom, cotyledon and hypocotyl explants.

[0050] In this example, the target organism for the adjuvant includes, but is not limited to, *Vibrio cholerae*, enterotoxigenic *Escherchia coli*. Other examples would include virus-like particles, for example, Norwalk virus capsid, and antigenic determinants of other pathogens, for example, bacterial, viral or parasitic.

[0051] EXAMPLE 3

[0052] The flowchart in Figure 12 depicts purification protocol of CTB-P1 fusion protein produced in *E. coli*. Resultant fractions from this protocol were resolved by SDS PAGE on the right panel. The following were placed in the first three lanes: Lane 1: molecular weight standards; Lane 2: a mixture of denatured (lower monomeric band) and non-denatured commercially available CT-B (pentameric upper band); Lane 3: whole cell extract from an IPTG-induced *E. coli*.

[0053] Following sonication and centrifugations, in step 70, extracts are separated into soluble (Lane 4) and insoluble (lane 5) fraction. The insoluble fraction, in step 72, is solubilized in 6.5 M urea and affinity purification on nickel column in step 74. The eluate (Lane 6) is more than 90% pure and can be subjected to dialysis promoting the refolding and oligomerization of the monomeric CTB-P1 fusion protein. By its mobility we conclude that the fusion protein can assemble into pentamers.

[0054] Finally, Eluate was dialyzed against PBS in step 76, and the purified, refolded CTB-P1 is shown in Lane 7. As noted in Lane 7, a CTB-P1 pentameter was produced. Additionally, a CTB-P1 monomer with an intramolecular disulfide bond was also produced.

[0055] Figure 13 demonstrates that the pentameric nature of the fusion protein allows it to bind to G_{M1} gangliosides. The ELISA plate was coated with GM1-ganglioside. On the left half of the plate, anti-CTB was used for detection. On the left half of the plate, anti-P1 was used for detection. CTB, CTB-P1 and P1 samples were applied to the plate as shown. The CTB is commercially available preparation of CTB and P1. CTB-P1 and P1 synthetic peptide are refolded samples purified as explained in Figure 12. Anti-CTB and anti-P1 are CTB- and P1-specific antibodies, respectively. These results demonstrate that the fusion is both able to retain its pentameric structure as well as its P1 epitope.

[0056] Various embodiments of the invention are described above in the Drawings and Description of Various Embodiments. While these descriptions directly describe the above embodiments, it is understood that those skilled in the art may conceive modifications and/or variations to the specific embodiments shown and described herein. Any such modifications or variations that fall within the purview of this description are intended to be included therein as well. Unless specifically noted, it is the intention of the inventor that the words and phrases in the specification and claims be given the ordinary and accustomed meanings to those of ordinary skill in the applicable art(s). The foregoing description of a

preferred embodiment and best mode of the invention known to the applicant at the time of filing the application has been presented and is intended for the purposes of illustration and description. It is not intended to be exhaustive or to limit the invention to the precise form disclosed, and many modifications and variations are possible in the light of the above teachings. The embodiment was chosen and described in order to best explain the principles of the invention and its practical application and to enable others skilled in the art to best utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. Therefore, it is intended that the invention not be limited to the particular embodiments disclosed for carrying out this invention, but that the invention will include all embodiments falling within the scope of the appended claims.

CLAIMS

What is claimed is:

1. A composition for enhancing immune response in an animal, comprising:

a peptide selected from the group SEQ. ID. NO: 1, SEQ. ID. NO: 3, SEQ. ID. NO: 4, SEQ. ID. NO: 5, SEQ. ID. NO: 6, SEQ. ID. NO: 7, and functional equivalents.

2. The composition of claim 1, further including a first antigen.

3. The composition in claim 2, wherein the first antigen is cholera toxin.

4. The composition in claim 2, wherein the peptide and first antigen comprise a fusion protein.

5. The composition of claim 1, wherein the composition is capable of mucosal administration.

6. The composition of claim 1, wherein the composition is a systemic adjuvant.

7. The composition of claim 1, wherein the composition is a mucosal adjuvant.

8. The composition of claim 1, wherein the composition is a epidermal adjuvant.

9. A method of enhancing immune response in an animal comprising:

administering a peptide selected from the group SEQ. ID. NO: 1, SEQ. ID. NO: 3, SEQ. ID. NO: 4, SEQ. ID. NO: 5, SEQ. ID. NO: 6, SEQ. ID. NO: 7, and functional equivalents, to the animal.

10. The method of claim 9, further including administering a first antigen to the animal.

11. The method of claim 10, wherein the first antigen is cholera toxin.

12. The method of claim 11, wherein the peptide and the first antigen comprise a fusion protein.

13. The method of claim 9, wherein the peptide is administered mucosally.

14. A method for delivering a cargo protein to an animal cell, comprising:

constructing a fusion protein including:

a peptide selected from the group SEQ. ID. NO: 1, SEQ. ID. NO: 3, SEQ. ID. NO: 4, SEQ. ID. NO: 5, SEQ. ID. NO: 6, SEQ. ID. NO: 7, and functional equivalents; and

a cargo protein, wherein the cargo protein is linked to the peptide; and

administering the fusion protein to the animal.

15. The method of claim 14, wherein delivering the fusion protein includes binding to the animal cell.

16. The method of claim 14, wherein delivering the fusion protein includes penetrating a membrane of the animal cell.

17. The method of claim 14, wherein the cargo protein is a first antigen.

18. The method of claim 17, wherein the fusion protein presents the first antigen to the immune system of the animal.

19. The method of claim 17, wherein the first antigen is a cholera toxin.

20. The method of claim 14, wherein the fusion protein is encoded by a DNA sequence capable of being incorporated into a viral DNA vector.

21. A genetically-modified living cell capable of enhancing immune response in an animal, comprising:

a first DNA sequence encoding a peptide selected from the group SEQ. ID. NO: 1, SEQ. ID. NO: 3, SEQ. ID. NO: 4, SEQ. ID. NO: 5, SEQ. ID. NO: 6, SEQ. ID. NO: 7, and functional equivalents.

22. The genetically-modified living cell of claim 21, further including a second DNA sequence encoding a first antigen.

23. The genetically-modified living cell of claim 22, wherein the first antigen is a cholera toxin subunit.

24. The genetically-modified living cell of claim 21, wherein the peptide is capable of enhancing a mucosal immune response in the animal.

25. The genetically-modified living cell of claim 22, wherein SEQ. ID. NO: 8 comprises the first DNA sequence and the second DNA sequence.

26. The genetically modified living cell of claim 22, wherein the first DNA sequence and the second DNA sequence code for SEQ. ID. NO: 9.

27. The genetically-modified living cell of claim 22, wherein the peptide is genetically fused to the first antigen.

28. A method for constructing a fusion protein for enhancing immune response in an animal, comprising:

constructing a vector including a first DNA molecule encoding for a peptide selected from the group SEQ. ID. NO: 1, SEQ. ID. NO: 3, SEQ. ID. NO: 4, SEQ. ID. NO: 5, SEQ. ID. NO: 6, SEQ. ID. NO: 7; and

linking the vector to a second DNA molecule encoding for a first antigen.

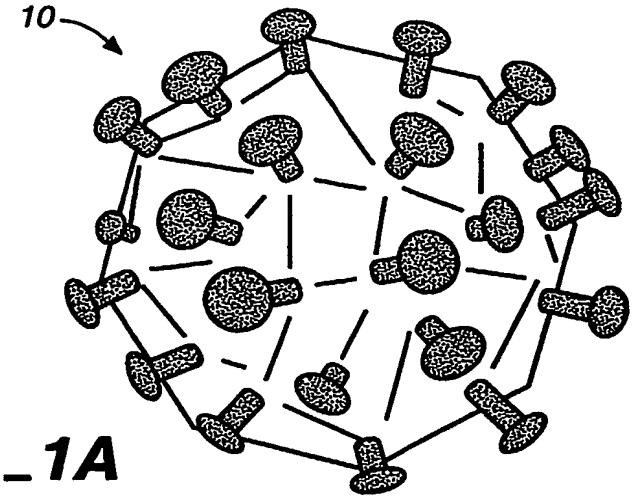


FIG._1A

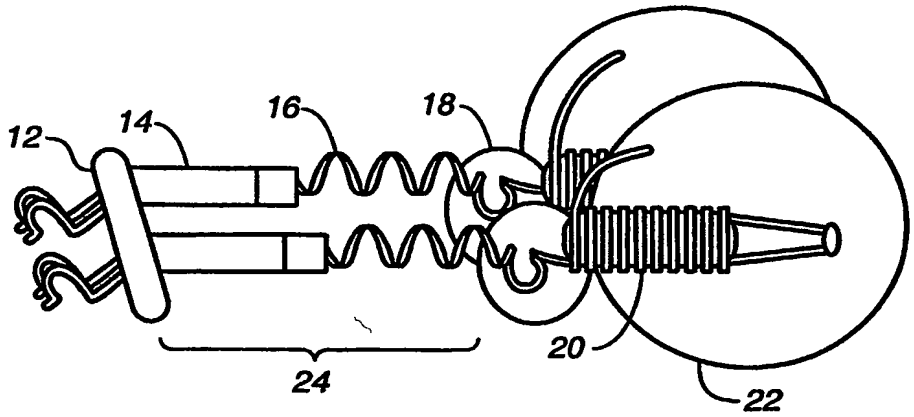


FIG._1B

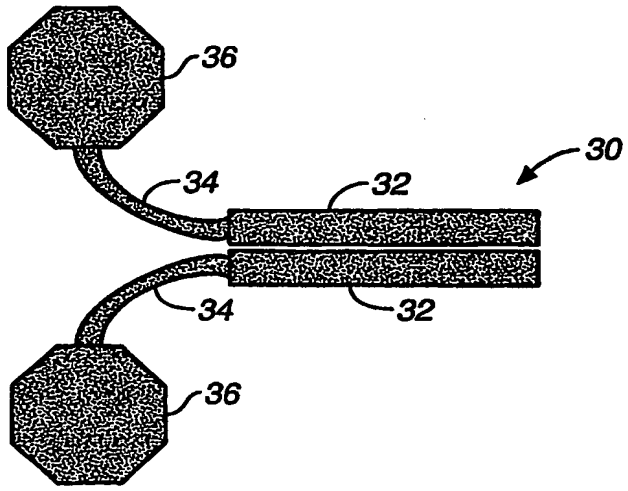


FIG._2

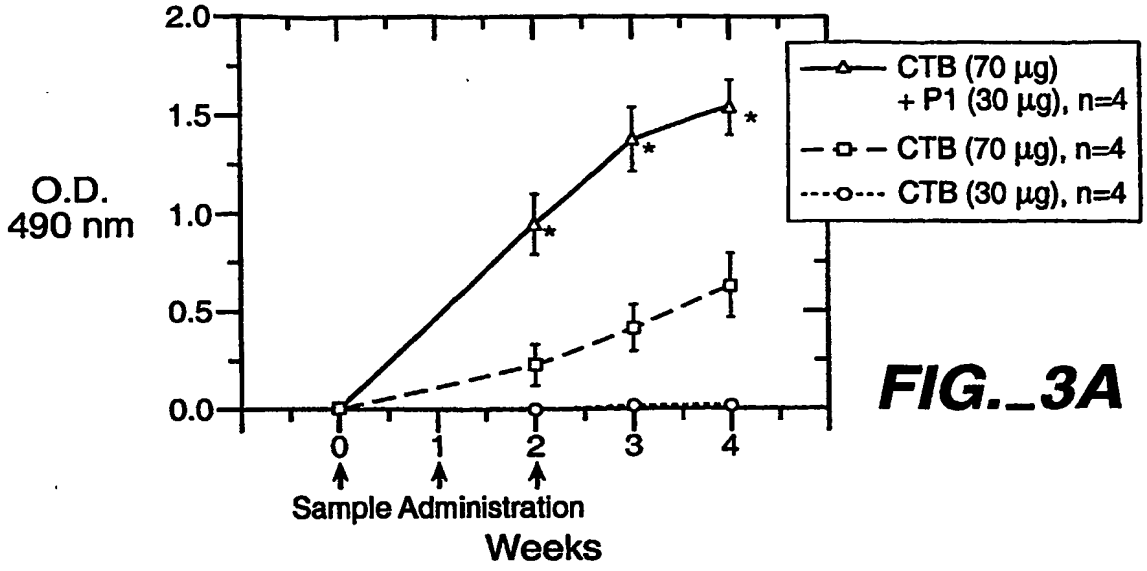


FIG._3A

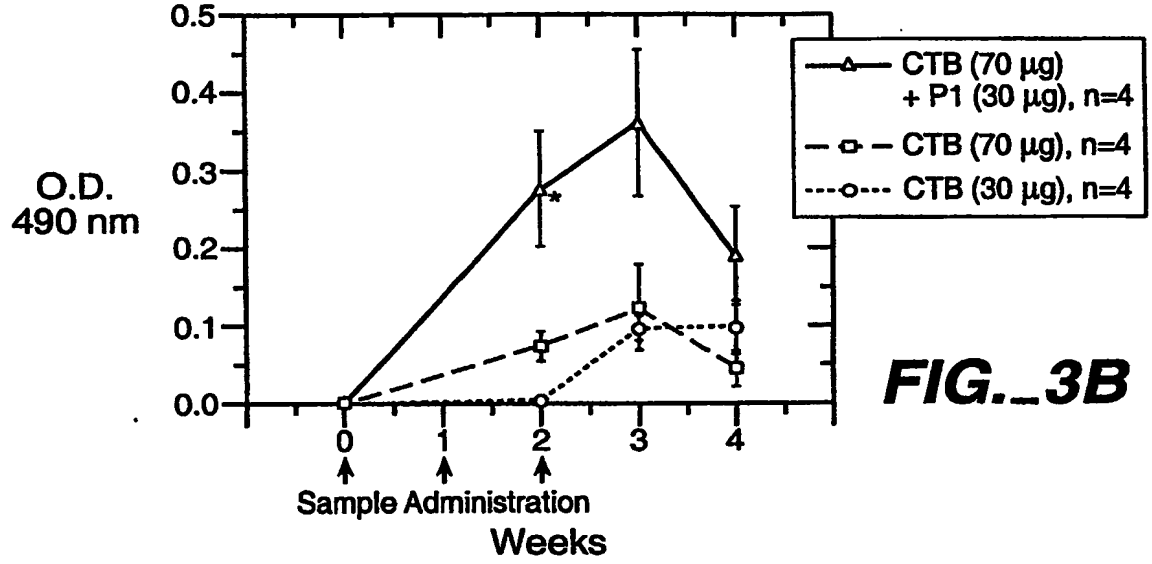


FIG._3B

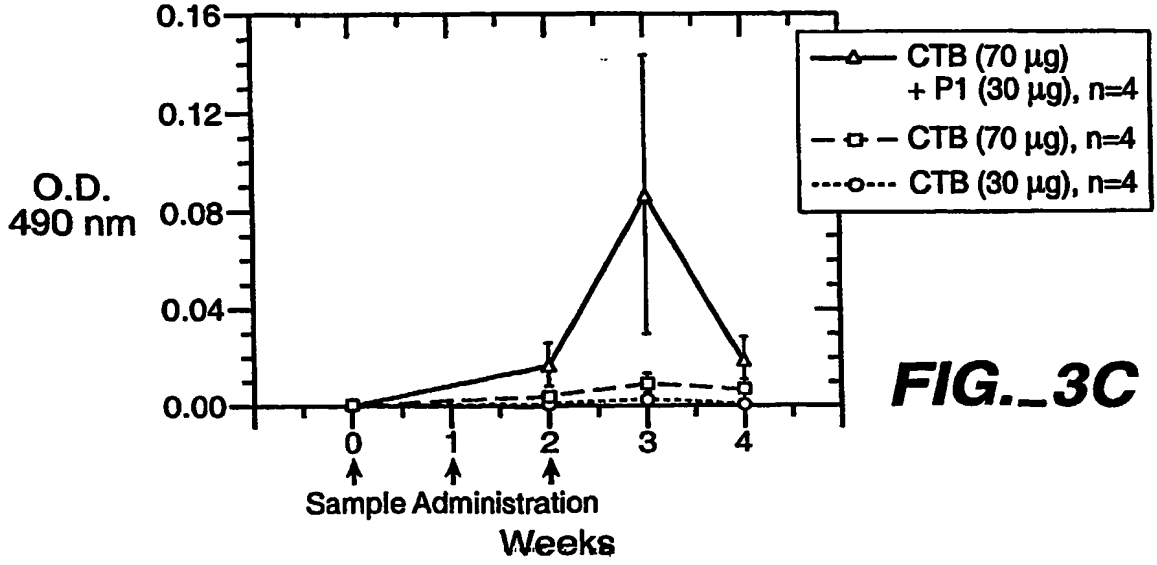


FIG._3C

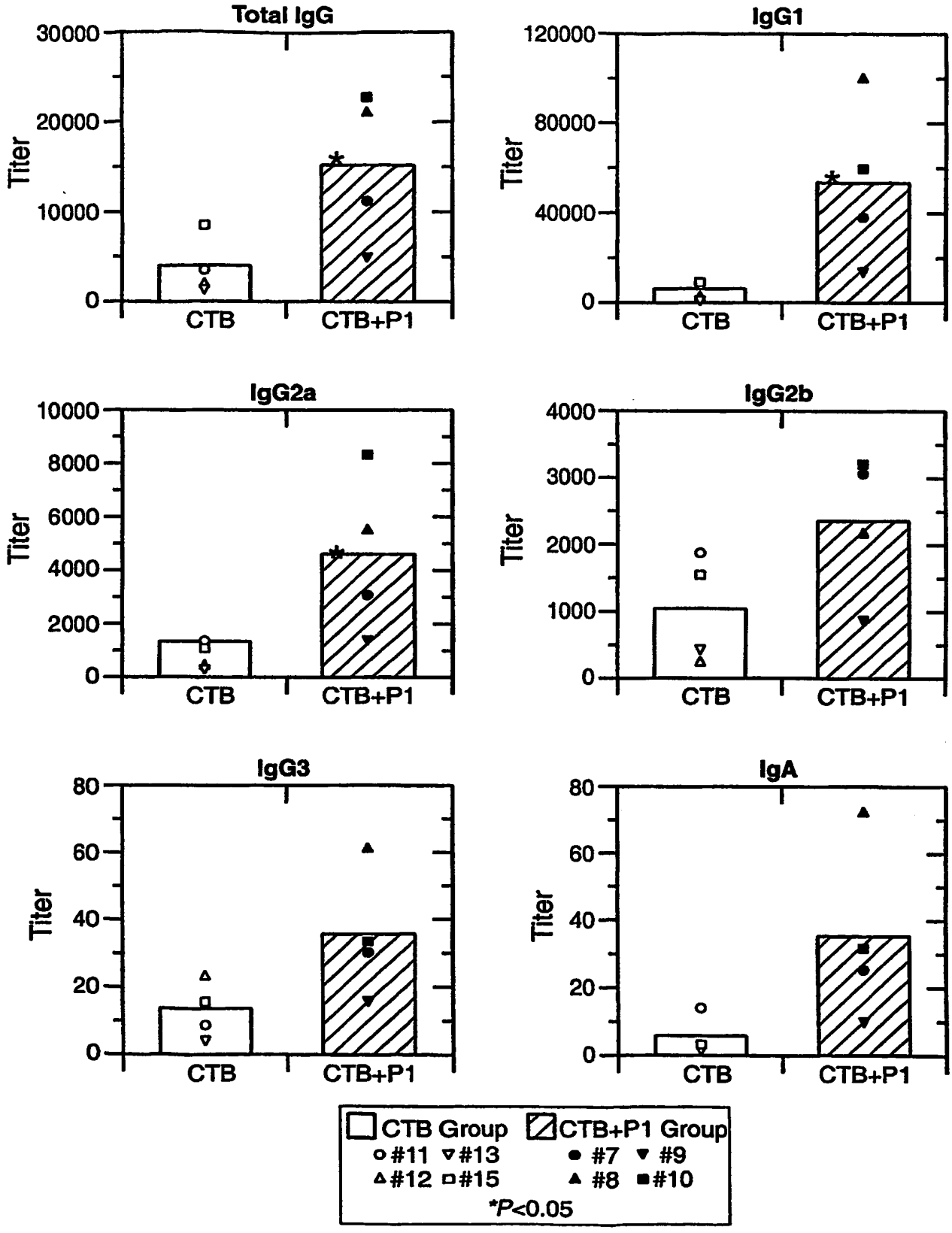

Total IgG
IgG1
IgG2a
IgG2b
IgG3
IgA
Titer
CTB
CTB+P1
CTB Group
CTB+P1 Group
#11 #13 #7 #9
#12 #15 #8 #10
*P<0.05

FIG. 4

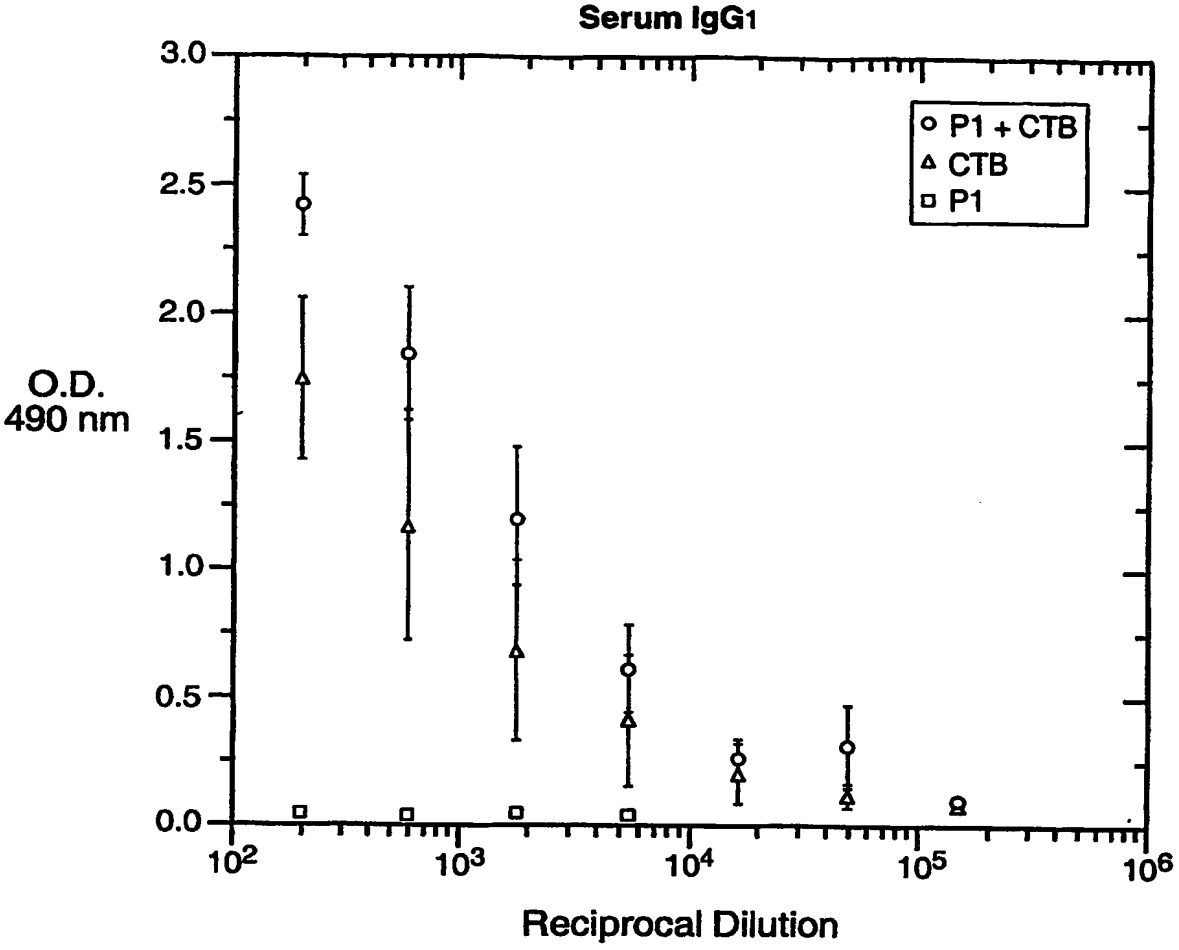

Serum IgG1
O.D.
490 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
P1 + CTB
CTB
P1
10^2
10^3
10^4
10^5
10^6
Reciprocal Dilution

FIG._5

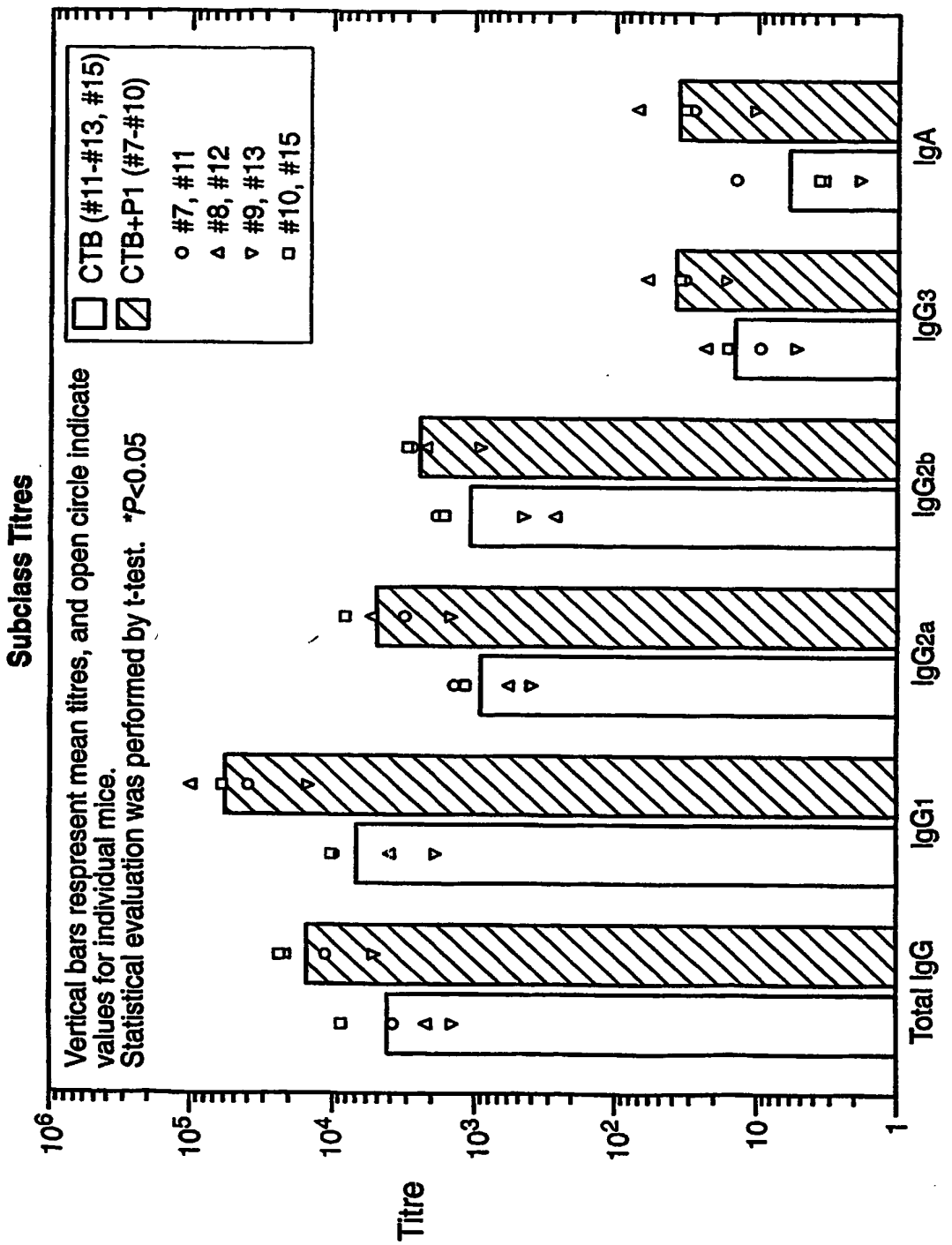

Subclass Titres
Vertical bars respresent mean titres, and open circle indicate values for individual mice.
Statistical evaluation was performed by t-test. *P<0.05
CTB (#11-#13, #15)
CTB+P1 (#7-#10)
#7, #11
#8, #12
#9, #13
#10, #15
Titre
10^6
10^5
10^4
10^3
10^2
10
1
Total IgG
IgG1
IgG2a
IgG2b
IgG3
IgA

FIG._6

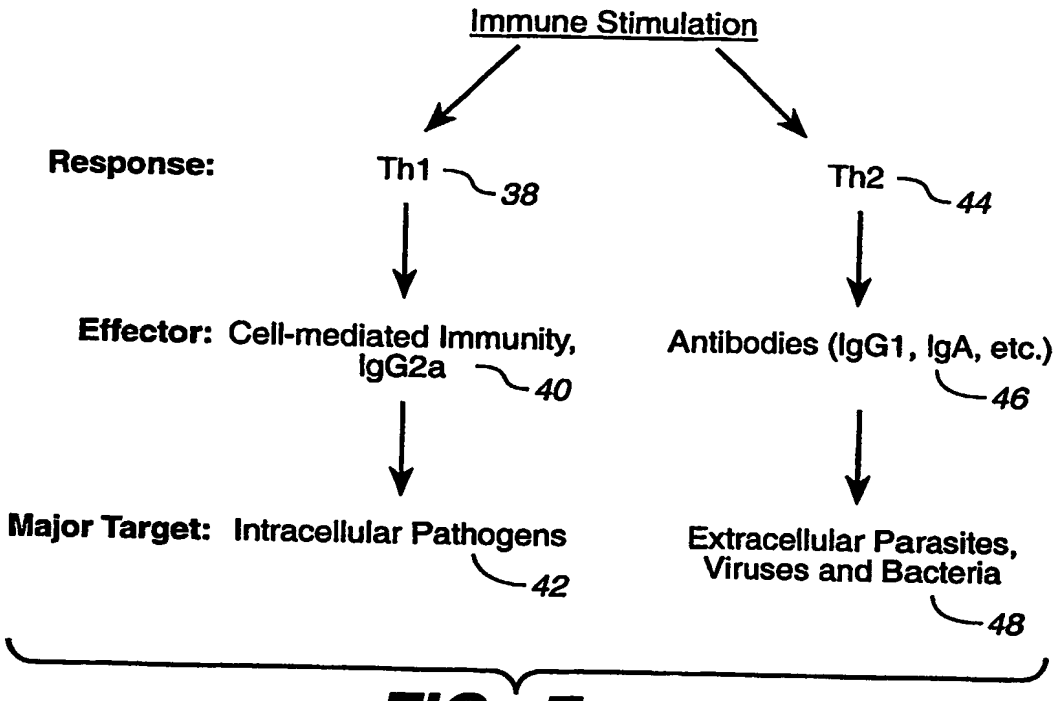

Immune Stimulation
Response:
Th1
38
Th2
44
Effector: Cell-mediated Immunity, IgG2a
40
Antibodies (IgG1, IgA, etc.)
46
Major Target: Intracellular Pathogens
42
Extracellular Parasites, Viruses and Bacteria
48

FIG._7

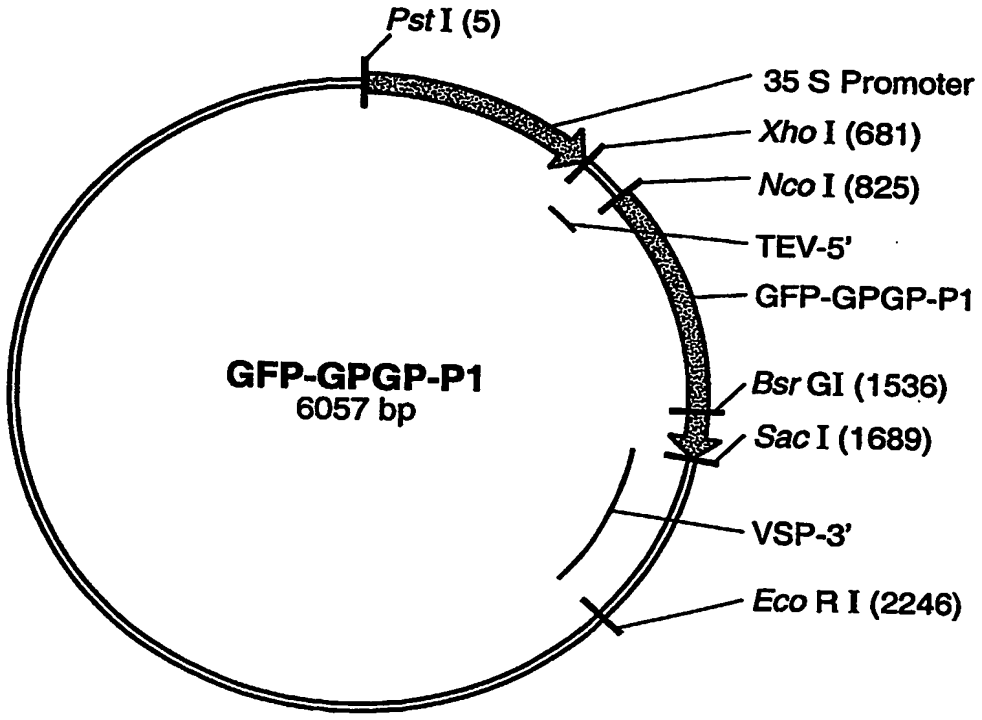

Pst I (5)
35 S Promoter
Xho I (681)
Nco I (825)
TEV-5'
GFP-GPGP-P1
GFP-GPGP-P1
6057 bp
Bsr GI (1536)
Sac I (1689)
VSP-3'
Eco R I (2246)

FIG._8

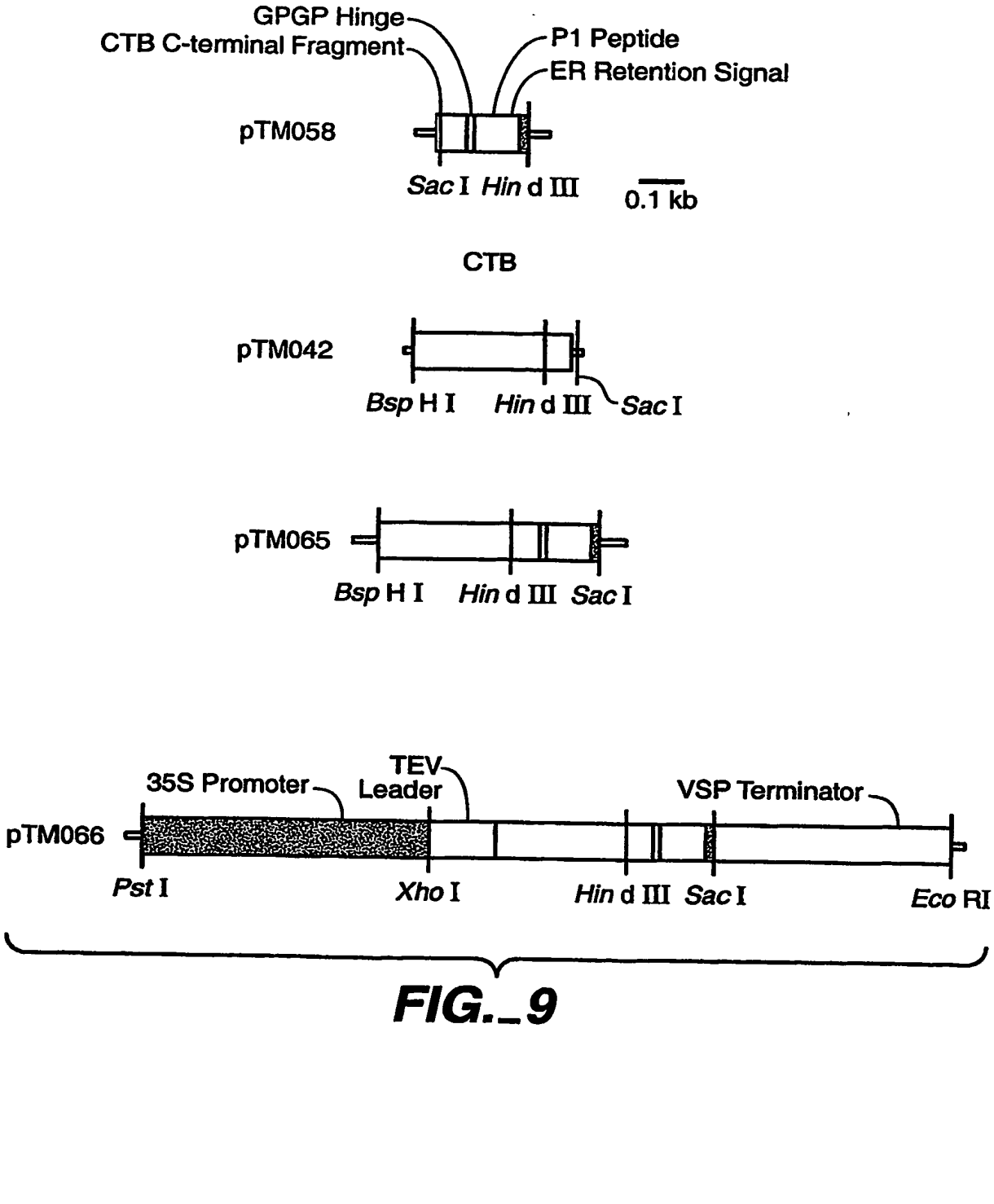

GPGP Hinge
CTB C-terminal Fragment
P1 Peptide
ER Retention Signal
pTM058
Sac I Hin d III
0.1 kb
CTB
pTM042
Bsp H I Hin d III Sac I
pTM065
Bsp H I Hin d III Sac I
35S Promoter
TEV Leader
VSP Terminator
pTM066
Pst I
Xho I
Hin d III Sac I
Eco RI

FIG._9

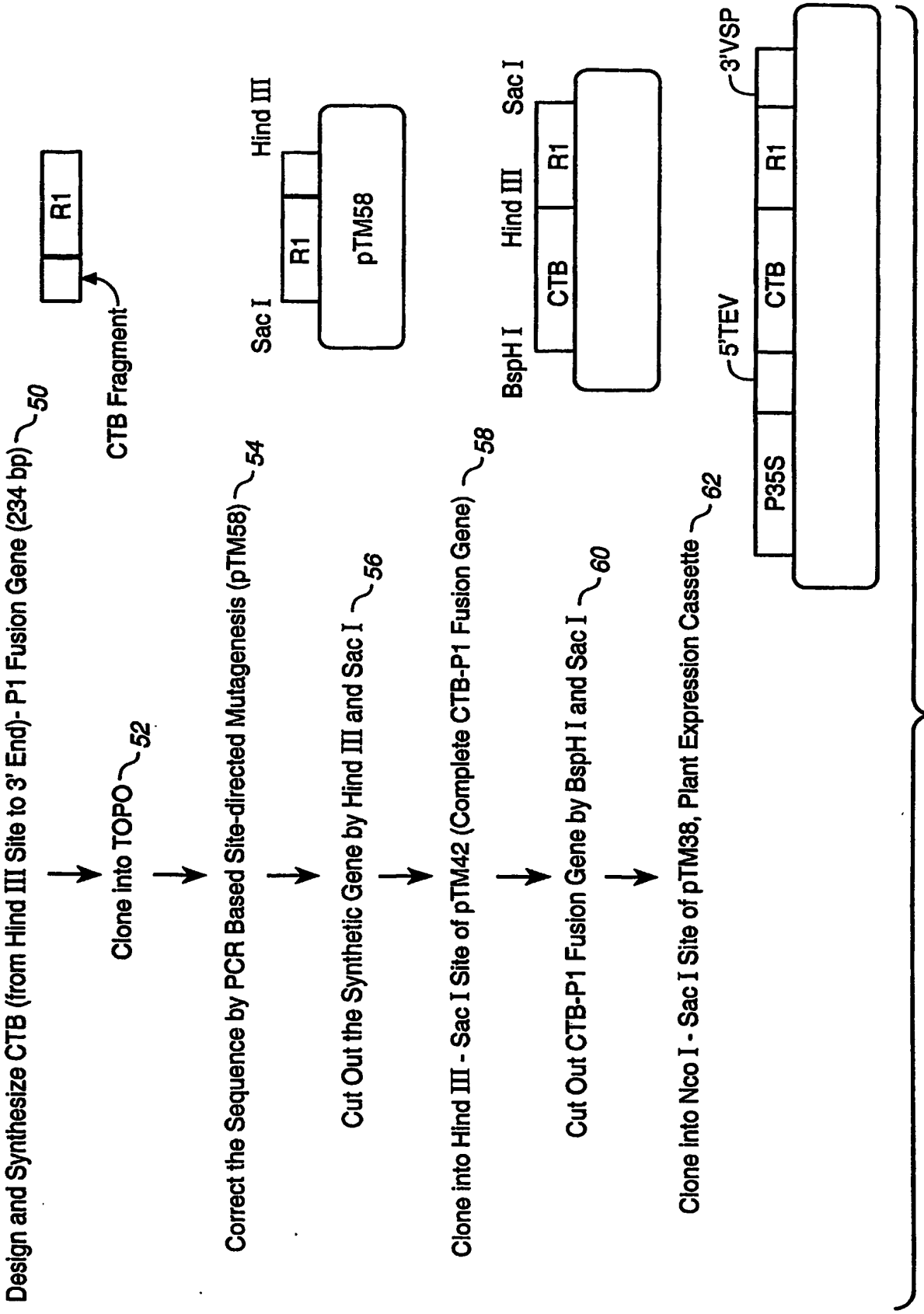

Design and Synthesize CTB (from Hind III Site to 3' End)- P1 Fusion Gene (234 bp)
50
R1
CTB Fragment
Clone into TOPO
52
Sac I
Hind III
R1
pTM58
Correct the Sequence by PCR Based Site-directed Mutagenesis (pTM58)
54
Cut Out the Synthetic Gene by Hind III and Sac I
56
BspH I
Hind III
Sac I
CTB
R1
Clone into Hind III - Sac I Site of pTM42 (Complete CTB-P1 Fusion Gene)
58
Cut Out CTB-P1 Fusion Gene by BspH I and Sac I
60
3'VSP
5'TEV
P35S
CTB
R1
Clone into Nco I - Sac I Site of pTM38, Plant Expression Cassette
62

FIG._10

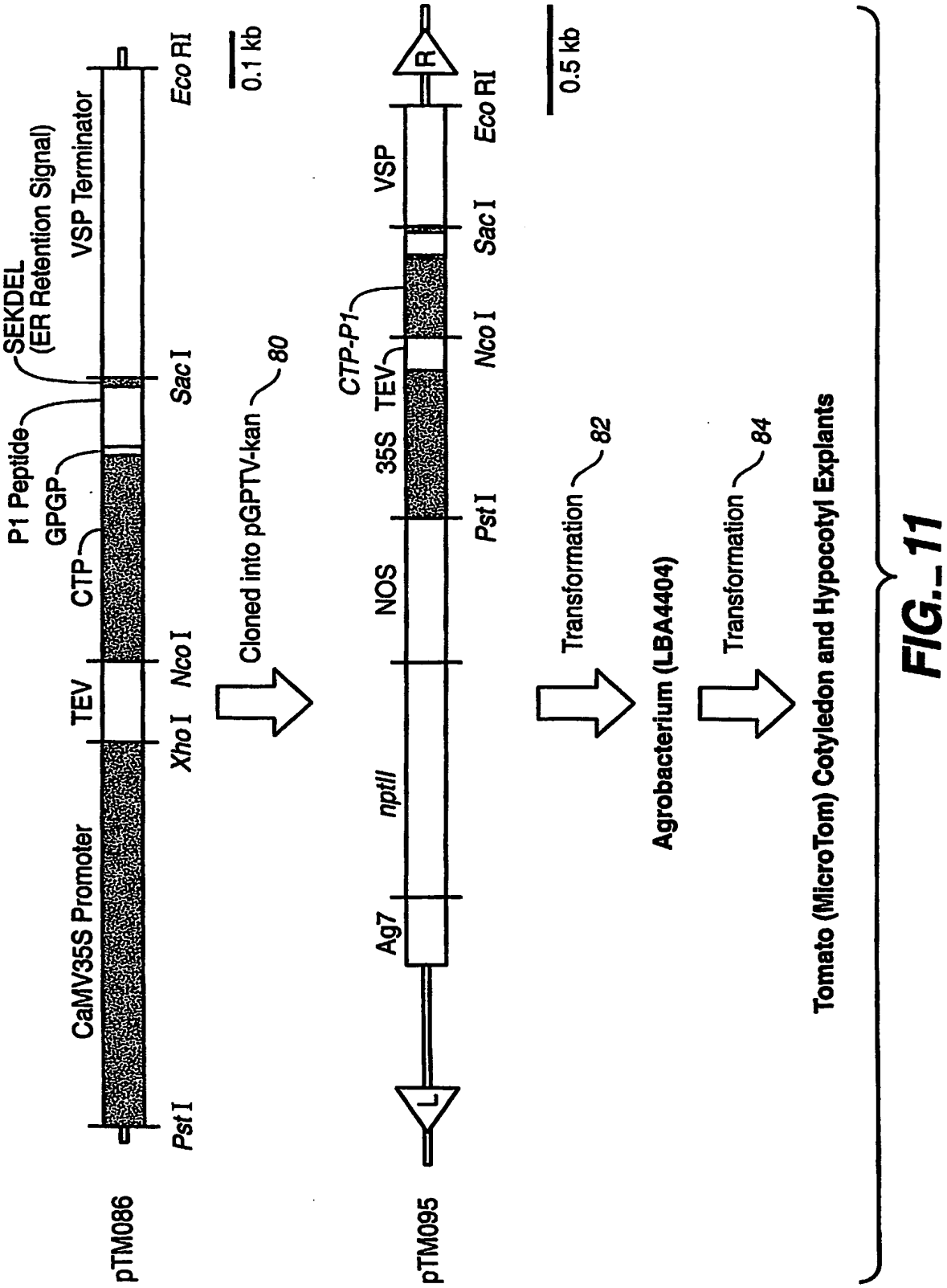
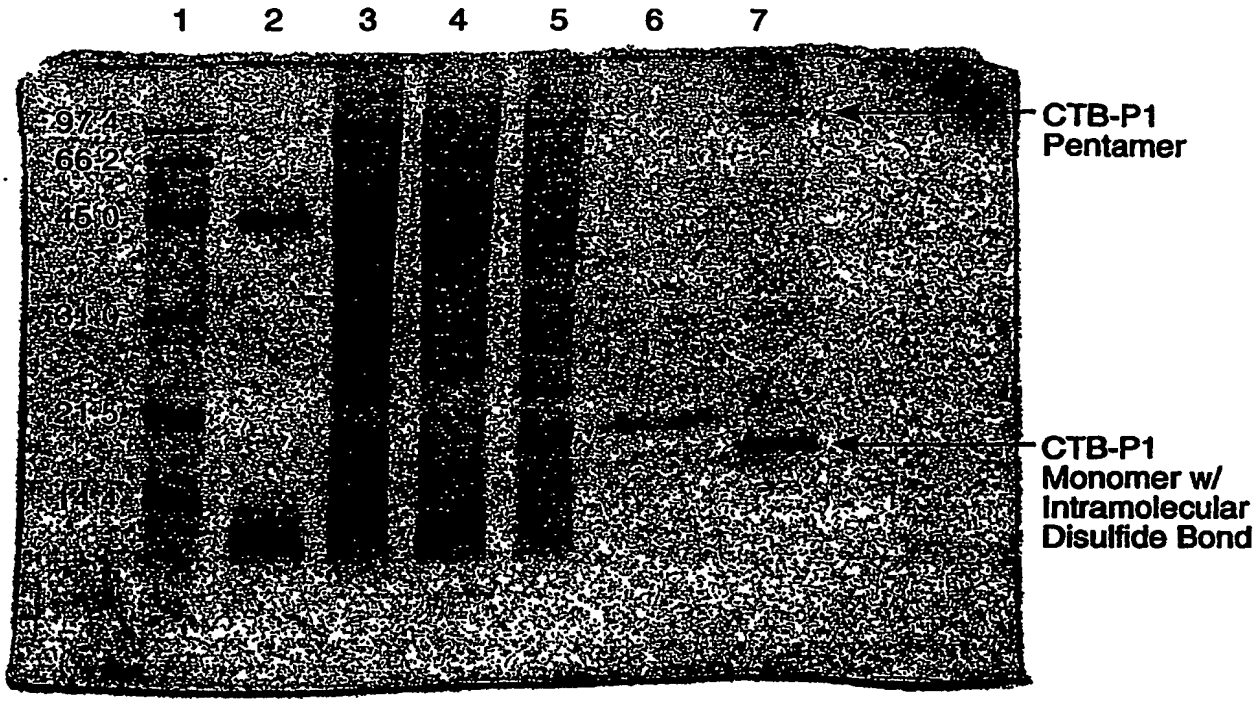

pTM086
Pst I
CaMV35S Promoter
TEV
Xho I
CTP
Nco I
GPGP
P1 Peptide
SEKDEL
(ER Retention Signal)
Sac I
VSP Terminator
Eco RI
0.1 kb
Cloned into pGPTV-kan
80
pTM095
L
Ag7
nptII
NOS
Pst I
35S
TEV
CTP-P1
Nco I
Sac I
VSP
R
Eco RI
0.5 kb
Transformation
82
Agrobacterium (LBA4404)
Transformation
84
Tomato (MicroTom) Cotyledon and Hypocotyl Explants

FIG._11



CTB-P1 Expressing E. coli Cells (Lane 3)

70 — Disrupted by Sonication

Soluable Fraction (Lane 4) Insoluable Fraction (Lane 5)

72 — Denatured and Solubilized w/ 6.5 M Urea

Nickel Column

74

Eluate (Lane 6)

76 — Dialyzed Against PBS

Purified, Refolded CTB-P1 (Lane 7)

FIG._12

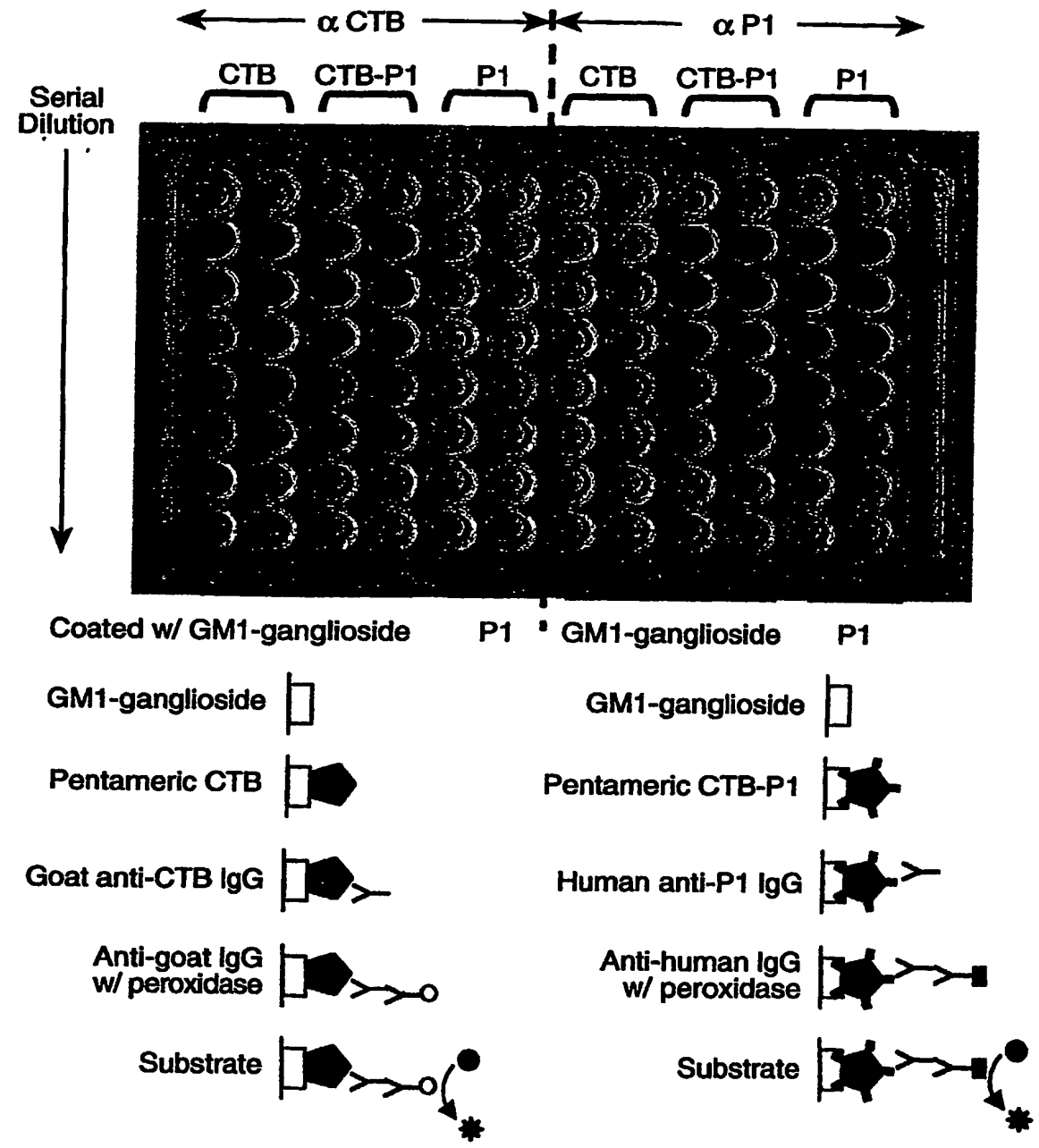


FIG._13

SEQUENCE LISTING

<110> Arizona Board of Regents, on behalf of Arizona Sta

<120> COMPOSITION AND METHOD FOR ENHANCING IMMUNE RESPONSE

<130> 130588.91361

<140> FILED HEREWITH
<141> 2003-03-06

<150> 60/362,247
<151> 2002-03-06

<160> 9

<170> PatentIn Ver. 2.1

<210> 1
<211> 35
<212> PRT
<213> Human immunodeficiency virus type 1

<220>
<221> PEPTIDE
<222> (1)..(35)
<223> HIV-1 gp41 peptide portion (residues 650-685)

<400> 1
Ser Gln Thr Gln Gln Glu Lys Asn Glu Gln Glu Leu Leu Glu Leu Asp
1 5 10 15

Lys Trp Ala Ser Leu Trp Asn Trp Phe Asp Ile Thr Asn Trp Leu Trp
20 25 30

Tyr Ile Lys
35

<210> 2
<211> 6
<212> PRT
<213> Human immunodeficiency virus type 1

<220>
<221> PEPTIDE
<222> (1)..(6)
<223> HIV-1 gp41 peptide portion (residues 663-668)

<400> 2
Glu Leu Asp Lys Trp Ala
1 5

<210> 3
<211> 36
<212> PRT
<213> Human immunodeficiency virus type 1

<400> 3
Cys Ser Gln Thr Gln Gln Glu Lys Asn Glu Gln Glu Leu Leu Glu Leu
1 5 10 15

Asp Lys Trp Ala Ser Leu Trp Asn Trp Phe Asp Ile Thr Asn Trp Leu
20 25 30

Trp Tyr Ile Lys
35

<210> 4
<211> 36
<212> PRT
<213> Human immunodeficiency virus type 1

<220>
<221> PEPTIDE
<222> (1)..(35)
<223> HIV-1 isolate MN clone V5 (residues 649-685)

<400> 4
Ser Gln Thr Gln Gln Glu Lys Asn Glu Gln Glu Leu Leu Gly Leu Asp
1 5 10 15

Lys Trp Glu Ser Leu Trp Asn Trp Phe Asp Ile Thr Asn Trp Leu Trp
20 25 30

Tyr Ile Lys Ile
35

<210> 5
<211> 36
<212> PRT
<213> Human immunodeficiency virus type 1

<220>
<221> PEPTIDE
<222> (1)..(36)
<223> HIV-1 isolate 593 clone (residues 649-685)

<400> 5
Ser Gln Asn Gln Gln Glu Lys Asn Glu Gln Glu Leu Leu Glu Leu Asp
1 5 10 15

Lys Trp Ala Gly Leu Trp Asn Trp Phe Glu Ile Thr Asn Trp Leu Trp
20 25 30

Tyr Ile Lys Ile
35

<210> 6
<211> 36
<212> PRT
<213> Human immunodeficiency virus type 1

<220>
<221> PEPTIDE
<222> (1)..(36)
<223> HIV-1 isolate 98BRRS012 (residues 649-685)

<400> 6
Ser Gln Asn Gln Gln Glu Lys Asn Glu His Glu Leu Leu Glu Leu Asp
1 5 10 15

Lys Trp Ala Asn Leu Trp Asn Trp Phe Asp Ile Thr Asn Trp Leu Trp
20 25 30

Tyr Ile Lys Ile
35

<210> 7
<211> 36
<212> PRT
<213> Human immunodeficiency virus type 1

<220>
<221> PEPTIDE
<222> (1)..(36)
<223> HIV-1 isolate 19242v3.20 (residues 649-685)

<400> 7

Ser Gln Asn Gln Gln Glu Lys Asn Glu Gln Asp Leu Leu Glu Leu Asp
1 5 10 15

Lys Trp Ala Ser Leu Trp Asn Trp Phe Asp Ile Ser Asn Trp Leu Trp
20 25 30

Tyr Ile Lys Ile
35

<210> 8
<211> 522
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CTB-P1 FUSION GENE

<400> 8
ccatggctat caagctcaag tttggagtgt tcttcactgt gctccttagc tctgcctatg 60
cacatggcac cccacaaaac atcactgact tgtgtgctga gtaccacaac acccaaatcc 120
acaaccctca atgacaagat ctttagctac accgagagcc ttgctggcaa gagggagatg 180
gctatcatcc cttcaagaat ggtgctacct tccaagtgga ggtgcctgga agccaacaca 240
ttgatagcca aaagaaggcc attgagagga tgaaggacac attaggatag cttacctcac 300
tgaggctaag gtggagaagc tttgtgtgtg gaacaacaag actccacatg ctattgctgc 360
cattagcatg gcaaatggtc ctggaccttc ccaaacccaa caagagaagā atgagcaaga 420
gcttttggag ttggacaagt ggcaagcctt tggaattggt ttgacatcac caattggctt 480
tggtatatca agatctctga gaaggatgaa ctctaagagc tc 522

<210> 9
<211> 171
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CTB-P1 FUSION PROTEIN

<400> 9
Met Ala Ile Lys Leu Lys Phe Gly Val Phe Phe Thr Val Leu Leu Ser
1 5 10 15

Ser Ala Tyr Ala His Gly Thr Pro Gln Asn Ile Thr Asp Leu Cys Ala
20 25 30

Glu Tyr His Asn Thr Gln Ile His Thr Leu Asn Asp Lys Ile Phe Ser

```
         35                  40

Tyr Thr Glu Ser Leu Ala Gly Lys Arg Glu Met Ala Ile Ile Thr Phe
     50                  55                  60

Lys Asn Gly Ala Thr Phe Gln Val Glu Val Pro Gly Ser Gln His Ile
 65                  70                  75                  80

Asp Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile
                 85                  90                  95

Ala Tyr Leu Thr Glu Ala Lys Val Glu Lys Leu Cys Val Trp Asn Asn
            100                 105                 110

Lys Thr Pro His Ala Ile Ala Ala Ile Ser Met Ala Asn Gly Pro Gly
        115                 120                 125

Pro Ser Gln Thr Gln Gln Glu Lys Asn Glu Gln Glu Leu Leu Glu Leu
    130                 135                 140

Asp Lys Trp Ala Ser Leu Trp Asn Trp Phe Asp Ile Thr Asn Trp Leu
145                 150                 155                 160

Trp Tyr Ile Lys Ile Ser Glu Lys Asp Glu Leu
                165                 170
```

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10/506796

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 September 2003 (18.09.2003)

PCT

(10) International Publication Number
WO 2003/075849 A3

(51) International Patent Classification[7]: A61K 39/12, 39/21, 39/02, 39/106, C12P 21/06, C12N 15/09, 15/31, 15/33, 15/40, 15/48

(21) International Application Number: PCT/US2003/007073

(22) International Filing Date: 6 March 2003 (06.03.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/362,247 6 March 2002 (06.03.2002) US

(71) Applicant *(for all designated States except US)*: **ARIZONA BOARD OF REGENTS** [US/US]; Arizona State University, P.O. Box 873511, Tempe, AZ 85287-3511 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **MOR, Tsafrir, S.** [IL/US]; 1508 E. Hermosa, Tempe, AZ 85282 (US). **MATOBA, Nobuyuki** [JP/US]; 200 East Southern Avenue Apt 116, Tempe, AZ 85282 (US). **ARNTZEN, Charles, J.** [US/US]; 7686 East Wilderness Trail, Superstition Mountain, AZ 85216-1806 (US).

(74) Agent: **MEIS, Christine, M.**; Quarles & Brady Streich Lang, LLP, One Renaissance Square, Two North Central Avenue, Phoenix, AZ 85004-2391 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to the identity of the inventor (Rule 4.17(i)) for the following designation US*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations*
— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *with international search report*

(88) Date of publication of the international search report: 22 July 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COMPOSITION AND METHOD FOR ENHANCING IMMUNE RESPONSE

(57) Abstract: A composition and method for enhancing immune response in a living organism is disclosed. In particular, the present disclosure provides an adjuvant peptide for use in raising an immune response to an antigen. The adjuvant peptide is selected from a group of peptides with an HIV-related sequence. Additionally, the adjuvant peptide can comprise a fusion-protein that acts as a mucosal adjuvant. The adjuvant peptide can be transformed into one or more living cells, such that the mucosal adjuvant can be produced in living cells and then administered by systemic, mucosal or epidermal delivery.

WO 2003/075849 A3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/07073

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 39/12, 39/21, 39/02, 39/106; C12P 21/06; C12N 15/09, 15/31, 15/33, 15/40, 15/48
US CL : 424/185.1, 186.1, 187.1, 188.1, 192.1, 201.1, 261.1; 435/69.1, 69.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/185.1, 186.1, 187.1, 188.1, 192.1, 201.1, 261.1; 435/69.1, 69.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Sequence search for SEQ ID NO: 1.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BACKSTROM. M. et al. Insertion of a HIV-1-neutralizing epitope in a surface-exposed internal region of the cholera B-subunit. Gene, November 1994, Vol. 149, No. 2, pages 211-217, see entire document.	1-13
Y	MACKSTROM. M. Characterization of an internal permissive site in the choleral toxin B-subunit and insertion of epitopes from human immunodeficeincy virus-1, hepatitis B virus and enterotoxigenic Escherichia coli. Gene. November 1995, Vol. 165, pages 163-171, see entire document.	1-13
Y	GEROGE-CHANDY.A. Cholera Toxin B Subunit as a Carrier Molecule Promotes Antigen Presentation and Increases CD40 and CD86 Expression on Antigen-Presenting Cells. INFECTION AND IMMUNITY. September 2001, Vol. 69. No. 9, pages 5716-5725, see entire document.	1-13
Y	US 6,271,198B1 (BRAISTED et al) 07 August 2001 (07.08.2001), see SEQ ID NO: 23.	1-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
29 September 2003 (29.09.2003)

Date of mailing of the international search report
25 NOV 2003

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Facsimile No. (703)305-3230

Authorized officer
Bao Qun Li
Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

PCT/US03/07073

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

The inventions listed as Groups I-IV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical feature of claimed invention drawn to a composition and use of the composition comprising a antigen epitope of HIV pepetide with a cholera toxin antigen is already known in the art as evidenced by Backstrom et al. (Gene 1994, Vol. 149, pp. 211-217). Backstrom et al. teach that non-toxin B-subunit of cholera toxin (CTB) is a powerful immunogen and a carrier for foreign peptides that is fused to the C- or N-terminal of CTB as a fusion protein. Backstrom et al. demonstrated that immunization of mice with the CTB:HIV hybrid protein elicited a high tittered serum Antibodies to CTB and also HIV gp120 epitope (See entire document, especially Abstract). Therefore, the special technical feature that linking all groups of inventions is lacking.

The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: different peptide antigens have different structures and functions and the adjuvant used for mucosal delivery differs from the adjuvant used for systemic delivery both in structure and function. Therefore, they are not linked together with same technical feature.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-13, drawn to a composition and a method for using the composition comprising a first antigen of cholera toxin and a peptide of HIV gp120, wherein the cholera toxin and peptide comprise a fusion protein.

Group II, claim(s) 14-20, drawn to a method for delivering a cargo protein.

Group III, claim(s) 21-27, drawn to a genetically modified living cell.

Group IV, claim(s) 28, drawn to a method for constructing a fusion protein.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

The species of peptide: 1). SERQ ID NO: 1, 2). SEQ ID NO: 3, 3). SEQ ID NO: 4, 4). SEQ ID NO: 5, and 5). SEQ ID NO: 7.

The species of adjuvant: A). Systemic adjuvant, B). A musocal adjuvant, and C). Epidermal adjuvant

The claims are deemed to correspond to the species listed above in the following manner:

Claims 1, 9, 14, 21 and 28 corresponding to species 1)-5).
Claims 6, 7 and 8 corresponding to species A) to C).

The following claim(s) are generic: 1, 6-9, 14, 21 and 28.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/07073

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-13 in the scope of SEQ ID NO: 1 and systemic adjuvant.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

PCT/US03/07073

Continuation of B. FIELDS SEARCHED Item 3:
MEDLINE, CAPLUS, WEST
search terms: HIV gp41, HIV envelope protein, HIV epitope, fusion protein, cholera toxin B, adjuvant